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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY 'S DOCKET NUMBER: 11146/10904

U S. APPLICATION NO. (If known, see 37 CFR 1 5): 10/088021

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US00/26438	26 September 2000 (26.09.00)	28 September 1999 (28.09.99)

TITLE OF INVENTION: THROMBOTIC EPISODE RISK ASSAYS USING OXIDIZED PHOSPHOLIPIDS

APPLICANT(S) FOR DO/EO/US: Naomi L. ESMON and Omid Safa JAMILABADI

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [ ] This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. [ ] The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is attached hereto (required only if not communicated by the International Bureau).
   b. [ ] has been communicated by the International Bureau.
   c. [X] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [ ] An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. [ ] is attached hereto.
   b. [ ] has been previously submitted under 35 U.S:C. 154(d)(4).
7. [X] Amendments to the claims of the International Aplication under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are attached hereto (required only if not communicated by the International Bureau).
   b. [ ] have been communicated by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [X] have not been made and will not be made.
8. [ ] An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. [ ] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [ ] An English lanugage translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [X] A FIRST preliminary amendment.
14. [ ] A SECOND or SUBSEQUENT preliminary amendment.
15. [ ] A substitute specification.
16. [ ] A change of power of attorney and/or address letter.
17. [ ] A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
18. [ ] A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. [ ] A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. [ ] Other items or information:

U.S. APPLICATION NO (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
10/088021	PCT/US00/26438	11146/10904

21. [X] The following fees are submitted:

	CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1040.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$740.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$710.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 890.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than [ ] 20 [X] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$ 130.00	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	56 - 20 =	36	x **$18.00**	$ 648.00	
Independent claims	4 - 3 =	1	x **$84.00**	$ 84.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$280.00**	$ 280.00	
TOTAL OF ABOVE CALCULATIONS =				$ 2032.00	
[X] Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2. +				$ 2032.00 [1016.00]	
SUBTOTAL =				$ 1016.00	
Processing fee of **$130.00** for furnishing the English translation later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				$	
TOTAL NATIONAL FEE =				$	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$	
TOTAL FEES ENCLOSED =				$ 1016.00	
				Amount to be refunded:	$
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a. [ ] A check in the amount of $ _____ to cover the above fees is enclosed.

b. [X] Please charge my Deposit Account No. 18-1260 in the amount of $ 1016.00 to cover the above fees. A duplicate copy of this sheet is enclosed.

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NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

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Karen L. Knezek
NAME

39,253
REGISTRATION NUMBER

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Naomi L. Esmon, et al.

For:	**THROMBOTIC EPISODE RISK ASSAY USING OXIDIZED PHOSPHOLIPIDS**
Application Serial No.	Unassigned
Filing Date:	Concurrently herewith
International Application No.:	PCT/US00/26438
International Filing Date:	26 September 2000

Assistant Commissioner for Patents
Box PCT
Washington, D.C. 20231

EXPRESS MAIL NO. EL861131911US DATE OF DEPOSIT 14 March 2002
I hereby certify that this correspondence is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Box PCT, Washington, D.C. 20231 on 14 March 2002
(Date of Deposit)

Derrick Gordon
Name of Depositor

Signature
Date of Signature: 14 March 2002

Dear Sir:

PRELIMINARY AMENDMENT

This preliminary amendment is being filed concurrently with the 35 U.S.C. §371 national stage filing of International Application No. PCT/US00/26438. In accordance with 37 C.F.R. §1.121(c)(3), this document implements changes to the claims by presenting an entire set of pending claims. An Appendix entitled Version With Markings to Show Changes Made is attached showing the current amendments to the claims in marked form. Any claim not accompanied by a marked up version should be construed as not having been changed relative to the immediate prior version thereof, if any.

IN THE CLAIMS

Please replace the previous version of the claims with the following clean version, wherein pending Claim 26 has been amended and new Claims 30-33 have been added.

1. In a coagulation assay for determining the propensity of patient risk for thrombotic disease wherein a phospholipid is employed as a reagent, the improvement comprising conducting said assay with an oxidized phospholipid reagent to obtain a first result and a non-oxidized phospholipid reagent to obtain a second result, and comparing said first and second result, and if said first result is prolonged in comparison to said second result, concluding that said patient is likely normal but if said first result is essentially the same as said second result, concluding that said patient likely has antibodies which block the function of oxidized phospholipids to a greater extent than unoxidized phospholipids.

2. A set of reagents for use in a coagulation assay consisting of a first reagent comprising one or more substantially non-oxidized phospholipids as determined by a ratio of the absorption bands at 233 and 215 nanometers, said first reagent maintained under oxidation-preventing gas and diluted with degassed buffer, and a second reagent comprising oxidized phospholipids prepared from a starting material of substantially non-oxidized phospholipids and subjected to controlled oxidation.

3. The set of reagents of Claim 2, wherein said phospholipids comprise phosphatidylethanolamine.

4. The set of reagents of Claim 3, wherein said phospholipids further comprise phosphatidlyserine.

5. The set of reagents of Claim 4, wherein said phospholipids further comprise phosphatidylcholine.

6. The set of reagents of Claim 5, wherein said phospholipids comprise 40% phosphatidylethanolamine, 20% phosphatidylserine and 40% phosphatidylcholine.

7. An assay to determine the presence of antibodies in a patient plasma sample, which antibodies selectively block the action of oxidized lipids, comprising:

(a) conducting a clotting assay by obtaining a first aliquot of said sample, providing activated protein C, providing an oxidized phospholipid reagent, initiating clotting and measuring the time of clotting to obtain a first clotting time;

(b) simultaneously or thereafter conducting a clotting assay by obtaining a second aliquot of said sample, providing activated protein C, providing an unoxidized phospholipid reagent, initiating clotting and measuring the time of clotting to obtain a second clotting time;

(c) comparing said first clotting time with said second clotting time and determining that the patient sample likely contains antibodies which block the function of oxidized lipids to a greater extent than unoxidized lipids if said first clotting time is essentially the same as said second clotting time.

8. The assay of Claim 7, further comprising obtaining baseline clotting values, said baseline clotting values obtained by measuring the clotting time of a third aliquot of said sample in the presence of an oxidized phospholipid reagent but without addition of activated protein C and obtaining a third clotting time baseline value, and measuring the clotting time of a fourth aliquot of said sample in the presence of a non-oxidized phospholipid reagent but without addition of activated protein C and obtaining a fourth clotting time baseline value, thereby determining if a given patient sample exhibits extended clotting time in the absence of activated protein C in comparison with a normal plasma sample, and concluding that said patient sample may have other components which may account for a prolonged clotting time when clotting time is tested in the presence of activated protein C according to steps (a) and (b).

9. The assay of Claim 7 or 8, wherein each of said phospholipid reagents comprise phosphatidylethanolamine.

10. The assay of Claim 9, wherein each of said phospholipid reagents further comprise phosphatidylserine.

11. The assay of Claim 10, wherein each of said phospholipid reagents further comprise phosphatidylcholine.

12. The assay of Claim 11, wherein each of said phospholipid reagents comprise 40% phosphatidylethanolamine, 20% phosphatidylserine and 40% phosphatidylcholine.

13. An assay to determine the propensity of a patient to have a thrombotic episode by measuring a first clotting time of a plasma sample taken from said patient in the presence of activated protein C and an oxidized phospholipid reagent , measuring a second clotting time of a plasma sample taken from said patient in the presence of activated protein C and an unoxidized phospholipid reagent, and analyzing the results, determining that said patient has a propensity for a thrombotic episode if said first clotting time is not prolonged as compared to said second clotting time.

14. The assay of Claim 13, wherein a patient immunoglobulin fraction is obtained from said plasma sample, and said immunoglobulin portion is utilized for said clotting time measurements.

15. The assay of Claim 13 or 14, further comprising diluting said plasma sample or immunoglobulin fraction thereof in an appropriate amount of normal plasma prior to measuring said first and second clotting times.

16. The assay of Claim 15, wherein said appropriate amount of normal plasma is about three parts for each one part of patient plasma sample.

17. The assay of Claim 15, wherein said appropriate amount of normal plasma is sufficient to make said immunoglobulin concentration about 0.6 mg/ml.

18. The assay of Claim 13 or 14, wherein each of said phospholipid reagents comprise phosphatidylethanolamine.

19. The assay of Claim 18, wherein each of said phospholipid reagents further comprise phosphatidylserine.

20. The assay of Claim 19 wherein each of said phospholipid reagents further comprise phosphatidylcholine.

21. The assay of Claim 20, wherein each of said phospholipid reagents comprise 40% phosphatidylethanolamine, 20% phosphatidylserine and 40% phosphatidylcholine.

22. The assay of Claim 15, wherein each of said phospholipid reagents comprise phosphatidylethanolamine.

23. The assay of Claim 22, wherein each of said phospholipid reagents further comprise phosphatidylserine.

24. The assay of Claim 23 wherein each of said phospholipid reagents further comprise phosphatidylcholine.

25. The assay of Claim 24 wherein each of said phospholipid reagents further comprise phosphatidylcholine.

26. The assay of Claim 16, wherein each of said phospholipid reagents comprise phosphatidylethanolamine.

27. The assay of Claim 26, wherein each of said phospholipid reagents further

comprise phosphatidylserine.

28. The assay of Claim 27 wherein each of said phospholipid reagents further comprise phosphatidylcholine.

29. The assay of Claim 28, wherein each of said phospholipid reagents comprise 40% phosphatidylethanolamine, 20% phosphatidylserine and 40% phosphatidylcholine.

30. The assay of Claim 17, wherein each of said phospholipid reagents comprise phosphatidylethanolamine.

31. The assay of Claim 30, wherein each of said phospholipid reagents further comprise phosphatidylserine.

32. The assay of Claim 31 wherein each of said phospholipid reagents further comprise phosphatidylcholine.

33. The assay of Claim 32, wherein each of said phospholipid reagents comprise 40% phosphatidylethanolamine, 20% phosphatidylserine and 40% phosphatidylcholine.

REMARKS

Prior to this Preliminary Amendment, Claims 1-29 as presented in the annex to the International Preliminary Examination Report were pending. With this Preliminary Amendment, Claim 26 has been amended to correct an improper multiple dependency and new Claims 30-33 have been added. Applicants respectfully request these changes be entered into the application.

Payment for the fees associated with these claims as amended has been directed on the Transmittal Letter to the United States Designated/Elected Office Concerning a Filing Under 35 U.S.C. §371 filed concurrently herewith.

Respectfully submitted,

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13 March 2002
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APPENDIX

VERSION WITH MARKINGS TO SHOW CHANGES MADE

The following is a marked-up version of the changes to claims which are being made in the attached Preliminary Amendment. Added material is underlined, and deleted material is bracketed.

IN THE CLAIMS

26. The assay of Claim 16[or 17], wherein each of said phospholipid reagents comprise phosphatidylethanolamine.

30. The assay of Claim 17, wherein each of said phospholipid reagents comprise phosphatidylethanolamine.

31. The assay of Claim 30, wherein each of said phospholipid reagents further comprise phosphatidylserine.

32. The assay of Claim 31 wherein each of said phospholipid reagents further comprise phosphatidylcholine.

33. The assay of Claim 32, wherein each of said phospholipid reagents comprise 40% phosphatidylethanolamine, 20% phosphatidylserine and 40% phosphatidylcholine.

[redacted]

THROMBOTIC EPISODE RISK ASSAYS USING OXIDIZED PHOSPHOLIPIDS

TECHNICAL FIELD OF THE INVENTION

This invention relates to the field of diagnostic blood clotting assays which are indicative of the risk of thrombotic episodes.

BACKGROUND OF THE INVENTION

It is well recognized that physiological blood coagulation requires the presence of membranes composed of negatively charged phospholipids. Zymogen activations occur rapidly when the enzyme, usually a vitamin K dependent protein, binds to a cofactor, usually a non-vitamin K dependent protein, to activate a substrate, usually a vitamin K dependent protein, reviewed in (Mann, K.G., Jenny R.J., and Krishnaswamy, S. 1988. "Cofactor proteins in the assembly and expression of blood clotting enzyme complexes," Ann. Rev. Biochem. 57:915-956; and Furie, B. and Furie, B.C. 1988. "The molecular basis of blood coagulation," Cell. 53:505-518). These reactions include those that are both procoagulant and those that are anticoagulant.

In addition to a net negative charge, the nature of the phospholipid head group appears to contribute to catalytic and binding efficiency. Phosphatidylserine (PS) is generally considered to be the most important phospholipid (Pei, G., Powers, D.D., and Lentz, B.R. 1993. "Specific contribution of different phospholipid surfaces to the activation of prothrombin by the fully assembled prothrombinase," J. Biol. Chem. 268:3226-3233; and Mann, K.G., Jenny R. J., and Krishnaswamy, S. 1988. Ann. Rev. Biochem. 57:915-956). It has been found that the presence of phosphatidylethanolamine (PE) or cardiolipin potently enhanced the rate of inactivation of factor Va by the activated protein C (APC) complex (Smirnov, M.D. and Esmon, C.T. 1994. "Phosphatidylethanolamine incorporation into vesicles selectively enhances factor Va inactivation by activated protein," C. J. Biol. Chem. 269:816-819). APC is a critical

natural anticoagulant required for preventing lethal thrombosis. The inactivation of factors Va and VIIIa by the APC complex is crucial in the regulation of coagulation, as evidenced by the severe clinical problems which are observed if this reaction is compromised (Esmon, C.T. and Schwarz, H.P. 1995. "An update on clinical and basic aspects of the protein C anticoagulant pathway," Trends Cardiovasc. Med. 5:141-148). The presence of unsaturated fatty acids in the phospholipid vesicles also has been found to enhance the inactivation of factor Va (Smirnov, M.D., Ford, D.A., Esmon, C.T., and Esmon, N.L. 1999. "The effect of membrane composition on the hemostatic balance," Biochemistry 38:3591-3598). A role for PE in factor VIII binding (Gilbert, G.E. and Arena, A.A. 1995. "Phosphatidylethanolamine induces high affinity binding sites for factor VIII on membranes containing phosphatidyl-L-serine," J. Biol. Chem. 270:18500-18505), tissue factor-factor VIIa activation of factor X (Neuenschwander, P.F., Bianco-Fisher, E., Rezaie, A.R., and Morrissey, J.H. 1995. "Phosphatidylethanolamine augments factor VIIa-tissue factor activity: enhancement of sensitivity to phosphatidylserine," Biochemistry 34:13988-13993) and prothrombin activation (Smeets, E.F., Comfurius, P., Bevers, E.M., and Zwaal, R.F.A. 1996. "Contribution of different phospholipid classes to the prothrombin converting capacity of sonicated lipid vesicles," Thromb. Res. 81:419-426; and Billy, D., Willems, G.M., Hemker, H.C., and Lindhout, T. 1995. "Prothrombin contributes to the assembly of the factor Va-factor Xa complex at phosphatidylserine-containing phospholipid membranes," J. Biol. Chem. 270:26883-26889) has also been reported. In these latter studies, the PE effects were different both qualitatively and quantitatively from those on the APC complex (See (Smirnov, M.D., Ford, D.A., Esmon, C.T., and Esmon, N.L. 1999. Biochemistry 38:3591-3598) for discussion).

One group of patients who are at increased risk for thrombotic diseases are those who have lupus anticoagulants, which are antibodies which bind to anionic phospholipids used in clotting assays based on the PTT (partial thromboplastin time) or APTT (activated partial thromboplastin time) techniques. See The Merck Manual (16th Ed. 1992) at 1225; J.E. Ansell, Handbook of Hemostasis and Thrombosis (Little, Brown & Co., Boston) at 19 (1986). Typical PTT test results for patients having the lupus anticoagulant are a prolonged clotting time that fails to correct with a 1:1 mixture of the patient's and normal

plasma, a normal or minimally prolonged PT (prothrombin time), and a nonspecific depression of those clotting factors measured by a PTT technique (Factors XII, XI, X and VIII). The lupus anticoagulant antibodies may also react with cardiolipin which can interfere with assays utilizing cardiolipin as a reagent. See The Merck Manual, supra. Anti-cardiolipin or anti-phosphatidylethanolamine antibodies can cross react with each other, but not interact with sufficient affinity to procoagulant phospholipids to be anticoagulants. Because of the specificity of the APC complex for phospholipids, such antibodies would selectively inhibit APC anticoagulant activity without influencing the coagulation tests performed in the absence of APC which are used to diagnose the presence of a "lupus anticoagulant."

Despite interference of the lupus anticoagulant antibodies with procoagulant phospholipid in clotting tests in vitro, persons with the antibodies have been reported to have an increased risk for thrombosis, either venous or arterial. Further, repeated spontaneous abortions in the first trimester of pregnancy have also been reported. Id. Patients have been treated with long term anticoagulant therapy to reduce the possibility of thrombosis, but no adequate technique has been developed for monitoring the effectiveness of such therapy. It should also be noted that other patients, who do not necessarily test positively for the lupus anticoagulant, may also be at risk for thrombotic disease due to the presence of antibodies not detected by current clotting tests. Further, not all persons who have the lupus anticoagulant or other risk factors have an identical propensity for thrombosis.

In order to attempt to identify patients at risk for thrombosis, standard clotting tests have been performed on patient plasma. Because anticoagulant therapy carries significant risk in some patients, it is highly desirable to determine whether patients are likely to benefit from such therapy. Additional testing has been suggested as described above where PTT and/or PT test results do not appear to be normal, such as repeating the test with added normal plasma or further addition of excess phospholipid. Previously, a technique was developed to differentiate among lupus patients and among others which

patients have the highest propensity to have a thrombotic incident. This method was disclosed and claimed in U.S. Patent No. 5,472,852, discussed infra.

Previously, we observed that at least a subpopulation of lupus anticoagulants can inhibit the APC anticoagulant activity more effectively than prothrombin activation (Smirnov, M.D., Triplett, D.T., Comp, P.C., Esmon, N.L., and Esmon, C.T. 1995. "On the role of phosphatidylethanolamine in the inhibition of activated protein C activity by antiphospholipid antibodies," J. Clin. Invest. 94:309-316). This difference is dramatically augmented by the presence of PE in the membrane bilayer (Smirnov, M.D., Triplett, D.T., Comp, P.C., Esmon, N.L., and Esmon, C.T. 1995. J. Clin. Invest. 95:309-316; and Rauch, J., Tannenbaum, M., Tannenbaum, H., Ramelson, H., Cullis, P.R., Tilcock, C.P.S., Hope, M.J., and Janoff, A.S. 1986. "Human hybridoma lupus anticoagulants distinguish between lamellar and hexagonal phase lipid systems," J. Biol. Chem. 261: 9672-9677).

In U.S. Patent 5,472,852 , entitled " Assay For Detection of Selective Protein C Inhibition by Patients" a method for determining the propensity of a patient to have a thrombotic incident was disclosed. A membrane source was utilized in the assay comprising an effective amount of phosphatidylethanolamine (PE) and an effective amount of phosphatidylserine (PS). Preferably, 10 to 50% PE and 5 to 50% of PS was utilized in the assay. Phosphatidylcholine (PC) could be used in the assay to make up any remaining percentage. In the assay disclosed and claimed in the '852 patent, patient and control plasma is assayed in the presence and absence of exogenous activated protein C (APC). By comparing the clotting times of samples with and without exogenous APC and optimal phospholipids, the risk of thrombotic disease can be assessed.

During the process of clot formation, leukocytes are recruited into the growing thrombus and become activated. These activated leukocytes have been reported to release potent oxidizing agents like hydrogen peroxide and superoxide, substances known to oxidize phospholipids. Malech, H.L. and Gallin, J.I. 1987. "Neutrophils in human diseases," N. Engl. J. Med. 317:687-694; McCord, J.M. 1985. "Oxygen-derived free radicals in postischemic tissue injury," N. Engl. J. Med. 312:159-163.

Although it has been established that phospholipids have an important role in coagulation, and coagulation assays such as discussed above, and some reports on specific parameters of the phospholipids have been reported as discussed above, there is a continuing need to improve assays for determining the risk of thrombotic disease by improving reagents used in such assays. In addition, although reports as to release of oxidizing agents from leukocytes have inferred that membrane oxidation may be involved in thrombosis, there has been no report or prediction of a differential effect of oxidation of lipids on APC activity.

It has now been found that oxidized phospholipids selectively enhance the anticoagulant properties of APC, with little impact on the clot-promoting reactions. It has been also been found that a subset of antiphospholipid antibodies selectively eliminate the oxidized lipid enhancement. An assay is herein disclosed which is useful for assessing the risk of thrombotic episodes by utilizing oxidized and nonoxidized phospholipids as separate reagents. The sample is tested for clotting by using each of the reagents in a parallel assay. The results are compared to those obtained with normal plasma to assess whether the sample plasma may contain indicators of thrombotic disease.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a graph depicting the effect of phospholipid oxidation on coagulation assays in the presence or absence of APC determined as a function of time of oxidation of the phospholipid. Assays were run on the following: PE:PS:PC with APC (closed square) and without APC (open square), and PS:PC with APC (closed circle) and without APC (open circle). Oxidation of PE containing vesicles dramatically enhanced the anticoagulant activity of APC.

DETAILED DESCRIPTION OF THE INVENTION

We have now found that lipid oxidation is a mechanism for making the thrombus less thrombogenic by enhancing the anticoagulant activity of the activated protein C ("APC") complex. As a result, patients having antibodies targeted to oxidized

phospholipids are likely unable to adequately prevent thrombus extension and are concomitantly more prone to deep vein thrombosis and arterial thrombosis. The specificity of these antibodies prevent the evolution of the thrombus from clot- promoting to clot-inhibiting.

Recent data has indicated that many of the antibodies identified by current assays are either partially or entirely targeted toward oxidized forms of either the lipid or protein antigen (see, for example, (Horkko, S., Miller, E., Dudi, E., Reaven, P., Curtiss, L.K., Zvaifler, N.J., Terkeltaub, R., Pierangeli, S.S., Branch, D.W., Palinski, W. et al. 1996. "Antiphospholipid antibodies are directed against epitopes of oxidized phospholipids. Recognition of cardiolipin by monoclonal antibodies to epitopes of oxidized low density lipoprotein," J. Clin. Invest. 98:815-825). Surprisingly, it has now been found that oxidized phospholipids, that include phosphatidylethanolamine (PE), further enhances the activity of APC in the factor Va inactivation complex. In addition, the ability of at least a class of lupus anticoagulant or anti-phospholipid antibodies to inhibit APC activity is augmented by the presence of oxidized phospholipid. Patient plasma may be screened by assaying it in a one stage assay in the presence and absence of activated protein C , according to the methodology described in U.S. Patent 5,472,852 which is herein incorporated by reference. The method of the '852 patent can be modified by using a membrane source comprising an effective amount of oxidized phospholipid which includes PE.

Additional procedures may be helpful to the assay of the invention or interpretation of data derived therefrom. It may be desirable to determine baseline clotting values for patient samples in the absence of APC, as set forth in Example 2, in order to better assess characteristics of particular samples which will be tested in the assay in the presence of APC, with oxidized phospholipid reagent as compared to non-oxidized phospholipid reagent. Another procedure which may be desirable is assaying clotting time of a purified subportion or fraction of the patient plasma which is the immunoglobulin. A procedure for purifying immunoglobulin from patient plasma or serum is provided in Example 3, and there are other purification procedures known to the art that could be employed as well. Another additional procedure which can be used, when using either patient plasma or

patient purified immunoglobulin as a test sample, is diluting the test sample in known normal plasma. An appropriate dilution to use in the assay of the present invention may be determined by standard techniques such as preparing serial dilutions of the test sample, conducting clotting assays set forth herein on the various dilutions, and determining which dilutions provide desirable assay conditions such as time of clotting and permit the operator to determine differences which may occur, so that such differences are not masked by possible high concentrations of various factors in the sample or unusually low concentrations (as may occur from a disease or course of medication). In addition, samples which are too dilute may not have detectable differences in the assay. A preferred dilution of patient plasma in normal plasma has been found to be about one part patient plasma to three parts normal plasma - an approximate 1:4 dilution. In case of immunoglobulin patient samples, a concentration of about 0.6 mg/ml has been found to be most preferred.

In the assay of the present invention, a reagent comprising appropriate oxidized lipids is utilized. Appropriate oxidized lipids comprise a quantity of PE, preferably in combination with other phospholipids, and most preferably in combination with phosphatidylserine (PS). In another embodiment, the reagent used in the assay of the present invention comprises PE and phosphatidylcholine (PC). Most preferably, a reagent useful in the present invention comprises PE, PS and PC. Preferably, a natural lipid source is utilized to make the reagent, but it is contemplated that synthetic lipids could also be utilized if vesicles made therefrom exhibit sufficiently similar behavior to vesicles made from natural-source lipids in assays measuring the clotting time of normal plasma.

Other lipid reagents with different compositions of phospholipids may be made in a similar manner by varying the proportions of phospholipids used. Preferably, the lipid reagents are used of the composition described in U.S. Patent 5,472,852. Specifically, a phospholipid component which comprises an effective amount of PE to provide a differential, detectable effect between normal (control) plasma and plasma from patients having a propensity for thrombotic episodes and an effective amount of PS to complement said PE in a clotting assay is employed. Preferably, the phospholipid component comprises from about 10% to about 50% PE, from about 5% to about 50% PS and the

remainder PC or any phospholipid which is zwitterionic and has no net charge at neutral pH. Preferably, the PS component is from about 5% to about 25% of the phospholipid component. In a most preferred embodiment, the phospholipid component of the membrane source comprises about 40% PE, about 20% PS and about 40% PC by weight of the phospholipid component.

The phospholipids utilized to make the lipid reagent should be substantially non-oxidized prior to use, and precautions should be taken during preparation to maintain the phospholipids in a non-oxidized state until it is desired to make an oxidized reagent. The choice of the commercial source of the phospholipids has been found to be one variable in the degree of oxidation present in the non-oxidized controls. For example, we have found that those purchased from Avanti Polar Lipids, Inc. are much less oxidized than those purchased from other sources, based on both supplier statements and the enhancement in APC activity observed after copper-catalyzed oxidation. There are several methods available to test phospholipids for oxidation. One method is to observe the phospholipids with the unaided eye to determine if the phospholipids appear to be discolored. Oxidized phospholipids appear off-color or yellow in comparison to non-oxidized phospholipids. This is especially true of PE and PS. In addition, a simple spectrophotometric test is available. The ratio of the absorption bands at 233 and 215 nanometers in the UV-spectrum can be measured according to the procedure of Klein, R.A., Biochim Biophys Acta, 210:486 (1970). A ratio of 0.02 corresponds to about 0.1% oxidation products according to the Klein method. For the lipid reagent useful in the invention, it is desirable to utilize substantially non-oxidized lipids which when used in a reagent and tested against results obtained when oxidized lipids are used as reagent, will demonstrate a significant difference in the clotting time of normal plasma, in the presence of APC, as seen in Table I.

Prevention of oxidation can be accomplished by maintaining the phospholipids under argon or nitrogen gas. Therefore, diluting the phospholipid into extensively degassed buffer, preferably a buffer pretreated with Chelex 100 resin (Biorad), vortexing under nitrogen or argon is a method of preserving lipids in a non-oxidized state.

Example 1: Method Of Preparing Non-Oxidized And Oxidized Phospholipid Reagents

A reagent useful in the assay of the present invention was prepared comprising 40% PE, 20% PS and 40% PC. The individual phospholipids derived from bovine brain (and therefore comprised of naturally-occurring fatty acids) were obtained from Avanti Polar Lipids, Inc. They were tested using the Klein spectrophotometric assay and found to be less than 1% oxidized. The lipids were mixed in the weight proportions indicated, dried under argon and lyophilized 3 hours to remove organic solvents. They were then reconstituted under argon, suspended by vortex in 0.15 M NaCl, 10 mM HEPES, pH 7.5 or 0.15 M NaCl, 20 mM Tris HCl, pH 7.4 to 5 mg/ml total lipid. Buffers were treated with Chelex 100 resin (BioRad) and saturated with argon before use. Other reagents with different compositions of phospholipids may be made in a similar manner by varying the proportions of phospholipids used.

Liposomes were prepared by extrusion through a 100 nm polycarbonate filter (Nucleopore). The non-oxidized liposomes so prepared can be used immediately or stored. The non-oxidized liposomes may be stored under argon until needed for the assay or until oxidation is desired, for up to about three days at room temperature. Because a quantity of non-oxidized liposomes is needed for comparative purposes in the assay of the invention, it may be desirable to add a stabilizing agent effective for preventing oxidation to a quantity of non-oxidized liposomes which will be used in the assay in non-oxidized form. Such stabilizing agents include EDTA and antioxidants such as BHT or equivalents thereof. In a specific example, 1mM EDTA was added to a preparation made in accordance with the method provided above to preserve liposomes in a non-oxidized state. For non-oxidized liposomes which are to be further processed to oxidized liposomes using the methods given below, it is undesirable to add stabilizing agents.

Oxidized liposomes for use in the assay of the present invention may be made by allowing exposure of non-oxidized liposomes to air or by employing an oxidizing reaction. It is preferred that an oxidizing reaction be used so that a degree of control may be applied to the process. For oxidation, 1 μl of 10 mM $CuSO_4$ was added to 1 ml of liposomes (200 μg/ml) to yield 10 μM $CuSO_4$ in a glass tube. The copper ion acts as a catalyst for air

oxidation of the phospholipids. The suspension was then vortexed at 37°C to introduce air into the solution. Other methods of oxidation, such as those discussed in references (Chatterjee, S.N. and Agarwal, S . 1988. "Liposomes as membrane model for study of lipid peroxidation," Free Radical Biology and Medicine 4:51-72; and Goni, F.M. and Alonso, A. 1989. "Studies of phospholipid peroxidation in liposomes," In CRC Handbook of Free Radicals and Antioxidants in Biomedicine, J. Miquel, Quintanilha, A. T., and Weber, H., editors. CRC Press, Inc., Boca Raton, FL. 103-122), may also be used as long as it is controlled so that the vesicles do not disintegrate, as determined by empirical observation. Oxidation by air alone can be used, but it is preferred to generate the oxidized lipids utilizing an oxidizing reaction exemplified above. In any of the methods, the reaction can be blocked as desired by adding a chelating agent effective for blocking the catalyst or an antioxidant such as BHT. The suitability of the oxidized lipids may be assessed by a preliminary titration test comprising a clotting assay. In the environment and using conditions known to one skilled in the art of clotting assay, one can determine whether prolonged clotting occurs in the presence of APC in the preliminary titration test as compared to clotting assays with non-oxidized lipids.

Example 2: Determination of Baseline Clotting Values in The Absence of APC And Comparison To Values In The Presence of APC

Coagulation assays were performed as one stage clotting assays using the ST4 coagulation instrument (Diagnostica Stago, Parsippany, NJ). For assays in which APC and/or immunoglobulin (normal or patient sample) were omitted, volumes were made up with buffer (0.1 M NaCl, 0.02 M Tris-HCl, pH 7.5, 1 mg/ml gelatin).

The effect of lipid oxidation on coagulation assays in the presence or absence of APC was determined as a function of time of oxidation of the phospholipid (Fig. 1). Pooled normal plasma (50 µl) was placed in the reaction vessels and the following reagents added to the final concentrations indicated in parentheses: Factor X-activating enzyme from Russell's viper venom (prepared as per C.T. Esmon, Dissertation, Washington University, St. Louis, 1973) or obtained from American Diagnostica, Greenwich, CN)

(0.1-0.15 nM, sufficient to yield a 30 sec clotting time in the absence of APC); Phospholipid (10 μg/ml); APC (0.2 μg/ml); buffer to bring the final volume to 200 μl. After 1 min incubation at 37°C, clotting was initiated with $CaCl_2$ (6.25 mM).

Neither the presence of PE nor 20 hours of copper catalyzed lipid oxidation had a significant effect on the clotting assay in the absence of APC. Oxidation of vesicles containing only PS and PC did not enhance the ability of the vesicles to support APC anticoagulant activity. Oxidation of PE containing vesicles dramatically enhanced the anticoagulant activity of APC.

Baseline clotting values for plasma test samples as compared to known normal plasma can be obtained by measuring clotting time in the absence of APC, using oxidized or non-oxidized lipids. This determination will account for the possible contribution of strong lupus anticoagulant activity or other unknown components in the patient sample to the prolongation of clotting time. If one determines that the clotting time of the patient sample in the absence of APC is several (e.g., greater than 5 seconds) seconds longer than the clotting time of normal plasma in the absence of APC, then it will be expected that the clotting time of the patient sample in the presence of APC, whether or not the lipid source is oxidized, will be much longer than that of normal plasma.

In addition, the methodology of Example 2 may be modified by utilizing one of the additional measures discussed, supra, such as dilution of the patient sample in normal plasma prior to assaying or purification of immunoglobulin from said sample as discussed in Example 3.

Example 3: Evaluation of Normal Plasma-Derived IgG Compared to IgG from Plasma of Patents with Diagnosed Clotting Disorders

Patients used in this study are described in Table I. Patients 1-7 were evaluated as follows. Double centrifuged citrated plasma was used for all coagulation assays and prepared according to the method of Sletnes, K.E., Gravem, K., and Wisloff, F. 1992. "Preparation of plasma for the detection of lupus anticoagulants and antiphospholipid

antibodies." Thromb. Res. 66:43-53. The presence or apparent absence of lupus anticoagulant was determined by a prolonged clotting time with a commercial PTT reagent (Staclot^R LA, Diagnostica Stago) in a 50:50 mixture of test plasma and normal plasma. Plasma with clotting times exceeding the 95th percentile of a previously established control population underwent additional testing. The diagnosis was confirmed by normalization or significant correction of the clotting time (≥8 seconds) in the presence of hexagonal (II) phase phospholipid. Anti-cardiolipin IgG and IgM were tested by commercial ELISA methods on serum samples (Quanta Lite aCL IgG, IgM, Inova Diagnostics, San Diego, CA). Antibodies to prothrombin, protein S and β2-glycoprotein I were analyzed by ELISA as previously described (Safa, O., Crippa, L., Della, V.P., Sabbadini, M.G., Vigano, D.S., and D'Angelo, A. 1999. "IgG reactivity to phospholipid-bound beta(2)-glycoprotein I is the main determinant of the fraction of lupus anticoagulant activity quenched by addition of hexagonal (II) phase phospholipid in patients with the clinical suspicion of antiphospholipid-antibody syndrome." Haematologica 84:829-838).

Patients 8-13 were evaluated for lupus anticoagulant activity using either previous reports from medical records (in the case of patients who were anticoagulated since registration) or a commercially available kit for the dilute Russell's viper venom assay (American Diagnostica, DVVT #810 and DVV Confirm #815). Anti-cardiolipin IgG and antibodies to prothrombin, protein S, β2-glycoprotein I and Annexin V were determined by standard ELISA techniques as described previously for β2-glycoprotein I (Merrill, J.T., Shen, C., Lahita, R.G., and Mongee, A.B. 1997. "High prevalence of antiphospholipid antibodies in patients taking procainamide." J. Rheum. 24:1083-1088).

Previously, we showed that plasma or immunoglobulin derived from at least a subset of lupus patients could inhibit APC function selectively in a phosphatidylethanolamine dependent manner. Smirnov, M.D., Triplett, D.T., Comp, P.C., Esmon, N.L., and Esmon, C.T. 1995. "On the role of phosphatidylethanolamine in the inhibition of activated protein C activity by antiphospholipid anitbodies." J. Clin. Invest. 95:309-316; and Smirnov, M.D., Ford, D.A., Esmon, C.T., and Esmon, N.L. 1999. "The effect of membrane composition on the hemostatic balance," Biochemistry 38:3591-3598).

Although normal precautions against oxidation were taken, including storage under argon and usage within one week of liposome preparation, the oxidation state of the phospholipids used in those studies was not specifically known.

To test whether the use of oxidized lipids affect the clotting time, IgG from a selection of lupus and thrombosis patients was tested for activity against APC on oxidized vs non-oxidized liposomes.

Immunoglobulin (IgG) was purified from thrombotic patients by standard procedures. Plasma or serum was precipitated with 50% NH_4SO_4. The precipitate was resuspended and dialyzed vs 0.1 M NaCl, 0.02 Tris-HCl, pH 7.5. This material was then applied to a protein G column (Amersham-Pharmacia), washed with buffer and eluted with 0.1 M glycine, pH 2.5. The eluate was immediately neutralized with 1 M Tris-HCl, pH 9 and dialyzed vs buffer. Immunoglobulin was added to the clotting assays to a final concentration of 0.6 mg/ml.

The results of these assays are given in Table I. As performed, this assay is relatively insensitive to lupus anticoagulant activity (≤5 sec prolongation), which permits simplification of the data presented, and only the elongation of clotting time in the presence of APC is shown. Patients 1-7 originated from a coagulation service but were also selected as lupus anticoagulant positive. Patients 8-13 with systemic lupus erythematosis originated from a rheumatology clinic. In both groups where thrombosis has occurred, immunoglobulin exhibiting oxidation specific inhibition of APC activity is seen (Patients 1, 2, 4,10). These immunoglobulins had little effect on APC anticoagulant activity on non-oxidized phospholipid. Patients in each group (Patients # 6, 7 and 12) showed decreased activity on both lipid preparations. In the case of Patient #7 and especially Patient #12, differential activity on oxidized vs non-oxidized lipid was essentially maintained.

Therefore, testing a patient plasma sample in an assay in the presence of APC, and obtaining a first clotting time by using an oxidized lipid reagent and a second clotting time by using an unoxidized lipid reagent provides a method to subclassify antibody functional

activity in patient plasma. If the first clotting time is essentially the same as the second clotting time, one can predict that the patient sample likely contains antibodies which block the function of oxidized lipids to a greater extent than unoxidized lipids. This data may be useful for predicting the propensity for thrombotic disease or recurrence of thrombotic episodes.

If the plasma is normal, or does not contain such antibodies, one would expect a longer clotting time with the use of an oxidized phospholipid reagent as compared to a non-oxidized phospholipid reagent. (See Table I, Norm 1 and Norm 2).

Table I. Characterization of Patients

Patient #[1]	Sex	History[2]	LA[3]	ACA[4]	Other Antibody Reactivities[5]	Clotting Time (sec) with Oxidized Phospholipids[6]	Clotting Time (sec) with Non-oxidized Phospholipids[7]
1	F	DVT; EM	+	+++ (IgG)	PrS, β2	34.1	33.7
2	F	DVT; 3 miscarriages	+	+++ (IgG)	Pt, PrS, β2	31.2	34.0
3	F	DVT; lupus-like syndrome	+	-	Pt	54.4	35.6
4	M	MI; ischemic stroke	+	+++ (IgG)	Pt, β2	35.2	34.0
5	F	polyarthritis	+	+ (IgM) +/- (IgG)	- Pt, PrS, β2 (+/-)	56.9	27.1
6	F	lupus-like demyelinating syndrome	+	+++ (IgG)	Pt, β2	25.0	28.7
7	F	DVT, SLE	+	+ (IgG)	Pt, β2	36.0	27.0
8	F	SLE	+	+++ (IgG)	Pt	82.0	37.1
9	F	SLE; DVT; 2 fetal losses	-	++ (IgG)	Pt, PrS	90.7	39.6
10	F	SLE; 2 DVTs; 3 fetal losses	+	-	Pt, PrS, β2	41.5	39.0
11	F	SLE; necrotizing vasculitis	+	-	PrS, β2 (+/-)	90.3	41.5
12	F	SLE; CVA; preeclampsia; phlebitis	+	++ (IgG)	Pt, AnV	56.8	30.1
13	F	4 fetal losses	-	+ (IgG)	Pt, PrS	92.8	39.8
Norm1						66.7	32.6
Norm2						99.0	44.0

[1] Norm1 = normal control for patients #1-7; Norm2 = normal control for patients #8-13

[2] DVT = deep vein thrombosis; EM = pulmonary embolism; MI = myocardial infarction; SLE = systemic lupus erythematosis; and CVA = cerebral vascular accident.

[3] LA = lupus anticoagulant; patients #1-7 based on Staclot® LA assay (available from Diagnostica Stago, Inc.); patients #8-13 based on dilute Russell's Viper Venom Time (dRVVT) assay for American Diagnostica.

[4] ACA = anti-cardiolipin anti-body; IgM was not measured in patients #8-13.

[5] All samples were tested in ELISA for reactivity to prothrombin (Pt), protein S (PrS) and β_2-glycoprotein I (β2). Only patients #8-13 were tested against annexin V (AnV).

[6] Assay results reported as clotting time measured with oxidized liposomes in the presence of APC minus the clotting time with oxidized liposomes in the absence of APC.

[7] Assay results reported as clotting time measured with non-oxidized liposomes in the presence of APC minus the clotting time with non-oxidized liposomes in the absence of APC

WE CLAIM:

1. In a coagulation assay for determining the propensity of patient risk for thrombotic disease wherein a phospholipid is employed as a reagent, the improvement comprising conducting said assay with an oxidized phospholipid reagent to obtain a first result and a non-oxidized phospholipid reagent to obtain a second result, and comparing said first and second result.

2. A reagent for use in a coagulation assay comprising an oxidized phospholipid.

3. The reagent of Claim 2, wherein said oxidized phospholipid comprises phosphatidylethanolamine.

4. The reagent of Claim 3, further comprising phosphatidylserine.

5. The reagent of Claim 4, further comprising phosphatidylcholine.

6. The reagent of Claim 5, wherein said reagent comprises 40% phosphatidylethanolamine, 20% phosphatidylserine and 40% phosphatidylcholine.

7. An assay to determine the presence of antibodies in a patient plasma sample, which antibodies selectively block the action of oxidized lipids, comprising:

(a) conducting a clotting assay by obtaining a first aliquot of said sample, providing activated protein C, providing an oxidized phospholipid reagent, initiating clotting and measuring the time of clotting to obtain a first clotting time;

(b) simultaneously or thereafter conducting a clotting assay by obtaining a second aliquot of said sample, providing activated protein C, providing an unoxidized phospholipid reagent, initiating clotting and measuring the time of clotting to obtain a second clotting time;

(c) comparing said first clotting time with said second clotting time and determining that the patient sample likely contains antibodies which block the function of

oxidized lipids to a greater extent than unoxidized lipids if said first clotting time is essentially the same as said second clotting time.

8. The assay of Claim 7, further comprising obtaining baseline clotting values, said baseline clotting values obtained by measuring the clotting time of a third aliquot of said sample in the presence of an oxidized phospholipid reagent but without addition of activated protein C and obtaining a third clotting time baseline value, and measuring the clotting time of a fourth aliquot of said sample in the presence of a non-oxidized phospholipid reagent but without addition of activated protein C and obtaining a fourth clotting time baseline value, thereby determining if a given patient sample exhibits extended clotting time in the absence of activated protein C in comparison with a normal plasma sample, and concluding that said patient sample may have other components which may account for a prolonged clotting time when clotting time is tested in the presence of activated protein C according to steps (a) and (b).

9. The assay of Claim 7 or 8, wherein each of said phospholipid reagents comprise phosphatidylethanolamine.

10. The assay of Claim 9, wherein each of said phospholipid reagents further comprise phosphatidylserine.

11. The assay of Claim 10, wherein each of said phospholipid reagents further comprise phosphatidylcholine.

12. The assay of Claim 11, wherein each of said phospholipid reagents comprise 40% phosphatidylethanolamine, 20% phosphatidylserine and 40% phosphatidylcholine.

13. An assay to determine the propensity of a patient to have a thrombotic episode by measuring a first clotting time of a plasma sample taken from said patient in the presence of activated protein C and an oxidized phospholipid reagent , measuring a second clotting time of a plasma sample taken from said patient in the presence of activated protein C and an unoxidized phospholipid reagent, and analyzing the results, determining

that said patient has a propensity for a thrombotic episode if said first clotting time is not prolonged as compared to said second clotting time.

14. The assay of Claim 13, wherein a patient immunoglobulin fraction is obtained from said plasma sample, and said immunoglobulin portion is utilized for said clotting time measurements.

15. The assay of Claim 13 or 14, further comprising diluting said plasma sample or immunoglobulin fraction thereof in an appropriate amount of normal plasma prior to measuring said first and second clotting times.

16. The assay of Claim 15, wherein said appropriate amount of normal plasma is about three parts for each one part of patient plasma sample.

17. The assay of Claim 15, wherein said appropriate amount of normal plasma is sufficient to make said immunoglobulin concentration about 0.6 mg/ml.

18. The assay of Claim 13 or 14, wherein each of said phospholipid reagents comprise phosphatidylethanolamine.

19. The assay of Claim 18, wherein each of said phospholipid reagents further comprise phosphatidylserine.

20. The assay of Claim 19 wherein each of said phospholipid reagents further comprise phosphatidylcholine.

21. The assay of Claim 20, wherein each of said phospholipid reagents comprise 40% phosphatidylethanolamine, 20% phosphatidylserine and 40% phosphatidylcholine.

22. The assay of Claim 15, wherein each of said phospholipid reagents comprise phosphatidylethanolamine.

23. The assay of Claim 22, wherein each of said phospholipid reagents further comprise phosphatidylserine.

24. The assay of Claim 23 wherein each of said phospholipid reagents further comprise phosphatidylcholine.

25. The assay of Claim 24 wherein each of said phospholipid reagents further comprise phosphatidylcholine.

26. The assay of Claim 16 or 17, wherein each of said phospholipid reagents comprise phosphatidylethanolamine.

27. The assay of Claim 26, wherein each of said phospholipid reagents further comprise phosphatidylserine.

28. The assay of Claim 27 wherein each of said phospholipid reagents further comprise phosphatidylcholine.

29. The assay of Claim 28, wherein each of said phospholipid reagents comprise 40% phosphatidylethanolamine, 20% phosphatidylserine and 40% phosphatidylcholine.

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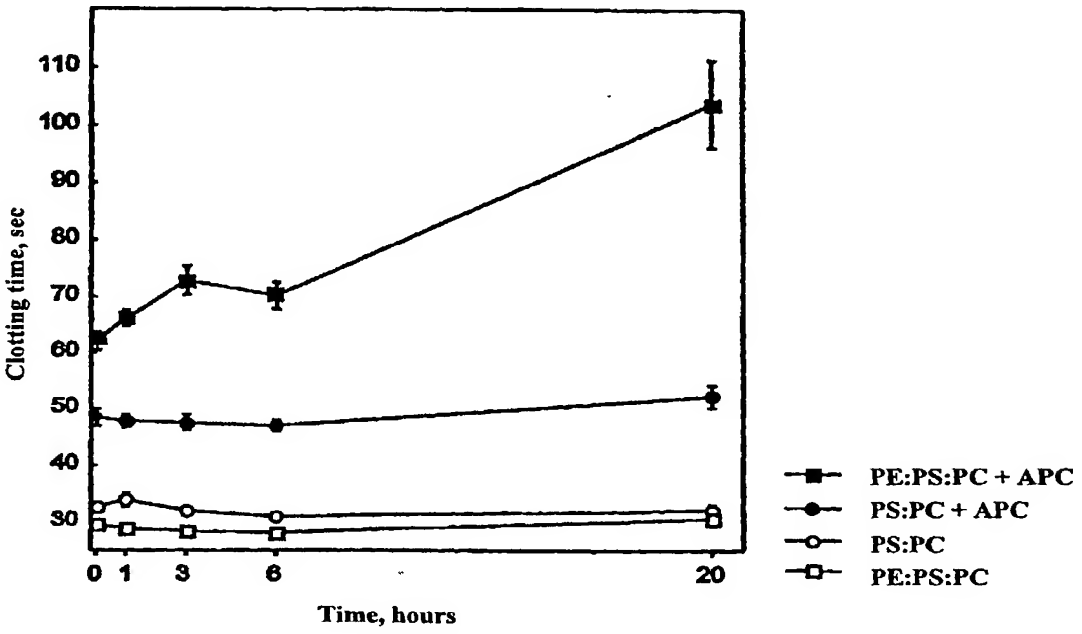

(57) Abstract: An assay to assess thrombotic risk in which oxidized lipids comprising phospholipids are utilized as a membrane source in a clotting assay and the results compared to an assay in which unoxidized phospholipid is used as a membrane source in the presence and absence of activated protein C ("APC"). The assay can monitor for the presence of antibodies in the patient which interfere specifically with the anticoagulant function of APC in an oxidation dependent or independent manner. This can indicate the propensity of the patient to experience episodes of vein thrombosis or arterial thrombosis.

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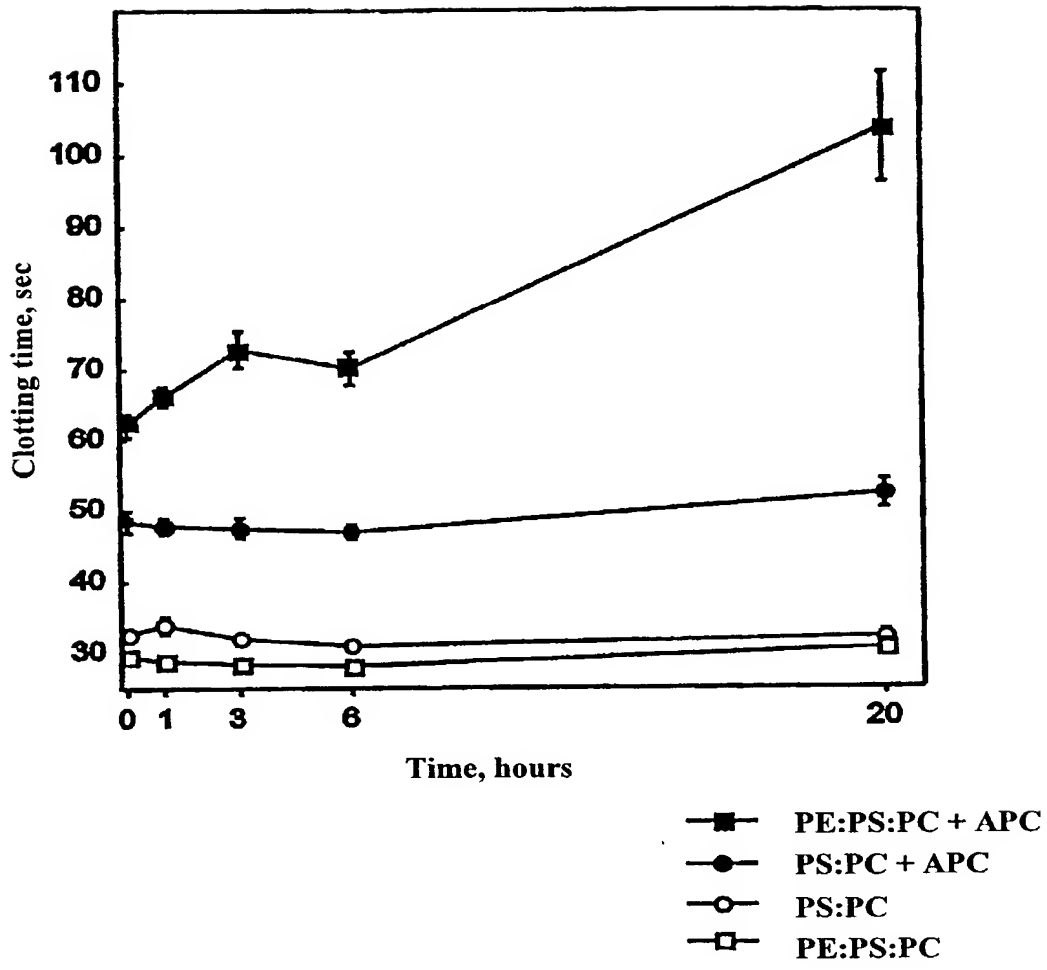
110
100
90
80
70
60
50
40
30
Clotting time, sec
0
1
3
6
20
Time, hours
PE:PS:PC + APC
PS:PC + APC
PS:PC
PE:PS:PC

Fig. 1

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For: **THROMBOTIC EPISODE RISK ASSAYS USING OXIDIZED PHOSPHOLIPIDS**

VERIFIED STATEMENT (DECLARATION)

CLAIMING SMALL ENTITY STATUS

(37 C.F.R. §§ 1.9(f) and 1.27(d))

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( ) the specification filed herewith.
(X) PCT International Application No. PCT/US00/26438, filed September 26, 2000 and under 35 U.S.C. §371 as Serial No. 10/088,021
( ) patent no. , issued .

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NAME OF PERSON SIGNING: Larry J. Kennedy

TITLE IN ORGANIZATION: V.P. Technology Transfer.

ADDRESS OF PERSON SIGNING: **825 Northeast 13th Street**
Oklahoma City, Oklahoma 73104

SIGNATURE Larry J. Kennedy

DATE July 15, 2002

DECLARATION AND POWER OF ATTORNEY

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name, that I believe that I am the original, first and sole inventor (if only one name is listed below) or I believe that we are the original, first and joint inventors (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention, design or discovery entitled **THROMBOTIC EPISODE RISK ASSAYS USING OXIDIZED PHOSPHOLIPIDS**, the specification of which (check one)

( ) is attached hereto; or
(X) was filed on **September 26, 2000**, as PCT International Application Number **PCT/US00/26438** and under 35 U.S.C. §371 as Serial No. **10/088,021**.

that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above; that I do not know and do not believe that said invention, design or discovery was ever known or used in the United States of America before my invention or discovery thereof, or patented or described in any printed publication in any country before my invention or discovery thereof, or more than one year prior to this application, or in public use or on sale in the United States of America more than one year prior to this application; that said invention, design or discovery has not been patented or made the subject of an inventor's certificate issued prior to the date of this application in any country foreign to the United States of America on an application filed by me or my legal representatives or assigns; and that I acknowledge the duty to disclose information of which I am aware which is material to the examiner of this application in accordance with 37 C.F.R. § 1.56(a).

I hereby claim foreign priority benefits under 35 U.S.C. § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application(s) for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

NUMBER	COUNTRY	DATE FILED	PRIORITY CLAIMED
N/A			

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below.

APPLICATION SERIAL NO.	DATE FILED
U.S. 60/156,564	September 28, 1999

I hereby claim the benefit under 35 U.S.C. § 120 of any United States Application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States Application(s) in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose material information as defined in 37 C.F.R. § 1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

APPLICATION SERIAL NO.	DATE FILED	STATUS
N/A		

I hereby appoint:

3 —	Eugenia S. Hansen	Reg. No. 31,966
	Karen L. Knezek	Reg. No. 39,253
	Rod A. Cooper	Reg. No. 42,436

all of the firm of Sidley Austin Brown & Wood LLP, my attorneys with full power of substitution and revocation, to prosecute this application and to transact all business in the United States Patent and Trademark Office connected therewith, and to file and prosecute any international patent applications filed thereon before any international authorities under the Patent Cooperation Treaty.

Send correspondence to:	Direct telephone calls to:
Eugenia S. Hansen Sidley Austin Brown & Wood LLP 717 N. Harwood, Suite 3400 Dallas, TX 75201	Eugenia S. Hansen (214) 981-3315 Atty. Docket No. **11146/10904**

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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